patients. However, vaccine efficacy is limited by effector cell dysfunction and increased presence of regulatory T cells characteristic of cancer patients. Ligation of CD3/CD28 delivers an antigen-independent stimulus to resting T cell populations. We postulated that stimulation with DC/tumor fusions followed by anti-CD3/CD28 would result in the significant expansion of activated T cells targeting tumor antigens. DCs were generated from adherent mononuclear cells cultured with rhIL-4, GM-CSF and TNFα and fused with RCC by coculture in 50% solution of polyethylene glycol. T cells were stimulated by DC/tumor fusions prior to or following exposure to antiCD3/CD28 antibody coated plates for 48 hours. A dramatic, statistically significant, increase in T cell proliferation was observed following the sequential exposure to DC/RCC fusions and anti-CD3/CD28 (SI 13.2). In contrast, stimulation by anti-CD3/CD28, DC/tumor fusions, or anti-CD3/CD28 followed by fusion cells did not result in significant T cell proliferation. Similarly, sequential stimulation by DC/RCC fusion cells followed by anti-CD3/CD28 resulted in a nearly 8 fold expansion of CD4+/CD25+ cells (n=10, p=0.001 compared to unstimulated T cells). A 16 fold increase in CD4/CD25/CD69+ cells was observed consistent with the expansion of activated T cells. In contrast, exposure to anti-CD3/CD28 alone or anti-CD3/CD28 followed by stimulation with fusion cells resulted in a 3 fold expansion of CD4/CD25+ T cells and a modest expansion of CD4/CD25/CD69+ cells. In concert with these findings, IFNγ production by CD4+ T cells was most pronounced following stimulation with DC/tumor fusions and expansion with anti-CD3/CD28 ($p<.01$). In 9 experiments, stimulation with DC/RCC fusions followed by expansion with anti-CD3/CD28 also resulted in a 5-fold and 4.6 fold expansion of CD4/CD25/Foxp3+ and IL-10 expressing T cells, respectively. In conclusion, we have demonstrated that stimulation of T cells by DC/RCC fusions followed by exposure to anti-CD3/CD28 antibodies results in the expansion of tumor reactive T cells that predominantly express markers of activation. We are developing a clinical trial in which patients will receive fusion/CD3/CD28 expanded T cells following in vivo depletion of regulatory T cells.

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γδ T CELLS AS IMMUNOTHERAPY FOR GLIOBLASTOMA MULTIFORME

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Background: Despite advances in therapy, the survival for glioblastoma multiforme (GBM) has remained unchanged for 50 years. We have investigated a cellular therapeutic therapy based on innate immune recognition of GBM using *ex vivo* expanded γδ T cells that are highly cytotoxic to GBM lines. Unlike αβ+CD8+ cytotoxic T lymphocytes, γδ T cells act directly against stress-associated antigens expressed on GBM and do not require MHC antigen recognition.

Methods: We examined the circulating γδ T cell number and function in healthy controls and patients at specific times; diagnosis, 1-14 days post resection, and 8-14 weeks post resection. Absolute lymphocyte and subset counts including γδ T cell counts were examined using flow cytometry. Functional response of γδ T cells was determined by proliferation in our clinically compliant expansion procedure and subsequent *in vitro* and *in vivo* cytotoxicity assays. Expansion cultures were harvested after two weeks and enriched for γδ T cells by immunomagnetic depletion of CD4+ and CD8+ T cells. Cytotoxicity of expanded γδ T cells was evaluated *in vitro* against GBM primary tumor cultures, established GBM cell lines and cultured astrocytes. *In vivo* assays were conducted in athymic nude mice against new and established luciferase-transduced human U251 GBM xenografts.

Results: At diagnosis, the circulating γδ T cell count is not significantly less than controls ($p = 0.12$). The absolute CD3 and CD4 count increase immediately after resection, likely due to removal of GBM-derived immunosuppressive cytokines. Surprisingly however, individual patients show a decrease in γδ T cell counts at this time. Expansion of γδ T cells from pre-resection GBM patients did not differ from controls ($p = 0.32$, n=4). although γδ T cell expansion was significantly impaired after cytoreductive therapy ($p = .007$, n=5). Expanded γδ T cells retain cytotoxicity against U251 and 1047 GBM cell lines and GBM primary cultures but spare normal astrocytes. Expanded γδ T cells slow the growth of U251 intracranial xenografts in athymic nude mice ($p = .008$) vs sham-treated controls. Preliminary observations suggest that growth of established U251 xenografts is slowed in selected animals as well.

Conclusions: GBM and therapy-induced immunosuppression present a formidable barrier to systemic cellular therapy. However, intracranial immunotherapy using expanded allogeneic γδ T cells represents a potentially effective immunotherapeutic strategy against GBM.

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SEMI-HIGH-DOSE AUTOLOGOUS AND A NON-MYELOABLATIVE REDUCED INTENSITY CONDITIONING ALLOGENEIC TRANSPLANTS INTEGRATED IN STANDARD OR DOSE DENSE THERAPY FOR BREAST CANCER: COST-EFFICIENCY ANALYSES SUPPORT DESIGN OF COST-EFFICIENT THERAPIES

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Introduction: DFS at 5 yr in LN+ BrCa depends on status at diagnosis and is on average 60% and in MBrCa 15%. HD-auto-Tx add significant DFS advantage of ~ 5% to 10% but statistically this is not a significant improvement of survival. In large series TRM of HD-auto-Tx is on average 3% in the adjuvant and up to 10% in the metastatic setting. Semi-HD is rarely associated with TRM. Allogeneic transplants (allo-Tx) induce an immune response in BrCa that may eradicate minimal residual disease if the Tx is administered in molecular remission. Based on these facts and existent collaborative patterns we designed in 2000 a treatment plan consisting of dose dense induction, two semi-HD-auto-Tx and a NMRICTx and decided to perform upfront cost computation.

Methods: In the Netherlands a cost system is used consisting of so named "diagnose behandelingscombinaties" (DBC) or diagnosis treatment combinations. In general costs are to be collected for each item or service independently. We used the therapeutic model described here above to compute the costs of treatment and used assumption based on literature data to define disease outcome at 5 yr for premenopausal women with ER+, her-2-neu negative BrCa. For each therapy median DFS was used to define outcomes. Disease outcomes were defined as alive without disease, alive with disease, death and toxic death. Utilities for treatment and outcome were derived from literature; The approach followed our Markov model design of 2001.

Results: Disease outcomes at 5 yr of LN + BrCa by respectively 4 cycles induction ChT (1), two semi-HD- auto-Tx (2) and a non-myeloablative reduced intensity conditioning Tx (3) with donor were anticipated to be 60%, 70% and 90% without and 15% with relapse. DBC were identified and included costs for first, second and third line ChT on outpatient basis, or with a hospital episode, costs of hormonal therapy, of a visit without procedure, of auto- and allo- stem cell mobilization and leukapheresis, of auto-and allo-Tx, of post Tx care, of follow-up, of terminal care. We adjusted for the fact that the DBC costs are based on HD- and ablative ChT whereas our approach uses semi-HD and NMRIC respectively. The computations of the cost per treatment with follow-up of five years showed that standard/dose dense ChT followed by hormonal therapy adds up to > 100,000 and that Tx types add each > 50,000 by use of adjusted rates for HD-/ablative Tx.

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THE ROLE OF HUMAN CYP2B6 POLYMORPHISM IN THE BIOACTIVATION OF CYCLOPHOSPHAMIDE USING CDNA EXPRESSED ENZYMES

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Cyclophosphamide (CPA) is used for the treatment of many tumors and as a part of the conditioning regimen prior to stem cell transplantation. CPA is a prodrug that is activated through cytochrome P450 2B6 (CYP2B6) in the liver. A high degree of inter- and intra-individual variation in CPA pharmacokinetics has been reported. We have investigated the formation of its active metabolite 4-hydroxycyclophosphamide (4-OH-CPA) in vitro using three different mutants of recombinant 2B6. The rate of formation of 4-OH-CPA was correlated to the enzyme activity for different mutations. Different concentrations of CPA (0-10 mM) have been incubated with microsomes from yeast cells expressing human CYP2B6*1 and the alleles *4, *6 as well as the point mutation G516T. The lowest K_m was observed in wild type 2B6 which also gave the lowest V_{max} (0.884 mM and 175 pmol/min/pmol P450, respectively). The ratio of V_{max}/K_m was 3-4 fold higher with CYP2B6*1 than with any of the mutants (*4, *6 and G516T). These results did not correlate with clinical data where patients, with hematological malignancies, having the CYP2B6 G516T mutation have been shown by Xie et al to have higher rates of CPA 4-hydroxylation. In vitro, however, all three mutant alleles were shown to have less CPA 4-hydroxylase activity than CYP2B6*1, possibly due to the high reductase activity of the cDNA-expressed CYP2B6*1 used as wild type for comparison.

In this paper, we propose that the discrepancy seen may depend on the ratio between the expressed levels of NADPH-cytochrome P450 reductase and cytochrome P450 in the cells used, especially in those expressing CYP2B6*1. Very high reductase to P450 ratios could enhance fast reactions, like CPA 4-hydroxylation, where the electron transfer may be rate limiting. The ratios in the microsomes used are not physiologically relevant and points out one difficulty in scaling from in vitro to in vivo. To evaluate the effect of the relative amount of reductase, we also used efavirenz as a slow substrate for CYP 2B6. Our results showed similar activity for the mutated enzymes for efavirenz compare the clinical data in HIV-1 patients, less enzyme activity of mutants G516T and *6 involved in efavirenz metabolism compare to wild type and also less activity was observed for *4. We concluded that the polymorphism of the CYP2B6 gene will influence the CPA metabolism and that it may be important to determine the genotype of the patients before starting treatment.

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TPO INDUCES HEMATOPOIETIC DIFFERENTIATION OF HUMAN ES CELLS

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Recently, it was demonstrated that TPO enhances hematopoietic differentiation of primate ES cells, but its role in differentiating human ES cells is unknown. Therefore, we sought to investigate the regulatory mechanism of TPO induced signals mediated by the c-mpl cytoplasmic domain during human embryonic stem (hES) cells hematopoietic commitment. We hypothesize that in human embryonic stem cells, binding of TPO to its c-mpl receptor causes three-dimensional alterations which bring the c-mpl cytoplasmic domain and Janus Kinase into close-proximity and thus induces the phosphorylation and dimerization of STAT5 molecule. Dimerized STAT5 molecules detach from the receptors and migrate to the nucleus where they bind GAS site and induce transcription of a set of target, hematopoiesis-related genes. NIH human ES cell lines (WI01) were used. In brief, to achieve EB formation, cells were incubated in differentiation medium, which consisted of knockout DMEM medium (GIBCO/BRL, Carlsbad, USA), supplemented with 20% non-heat-inactivated fetal bovine serum (FBS, Hyclone, USA), 1% nonessential amino acids, 1 mM L-glutamine, and 0.1 mM β-mercaptoethanol. Subsequently, DMEM was replaced by IMDM (GIBCO/BRL, USA) with the same supplements and additional two cytokines (100 ng/mL SCF and 100 ng/mL Flt-3 ligand (Flt-3L)) (control group). To investigate the role of TPO and VEGF, cells were additionally treated with 100 ng/mL TPO alone or in combination with 100 ng/mL rhVEGF. All cytokines were from the R&D systems (USA). There was a significant increase in the numbers of embryoid bodies (EBs) formation in TPO ($125/10^5$), TPO/VEGF ($150/10^5$ cells) when compared to controls ($10/10^5$ planted ES cells). This corresponded to the increase in CFU-C and specific progenitors (CD31/CD34 positive) and in hematopoietic (CD34-positive cells) cells. Analysis of gene expression during hematopoietic development demonstrated that TPO/VEGF combination increased mRNA expression of the TPO receptor (TPO-R) and VEGF (VEGF-R) receptors in hematopoietic progenitors as well as CD34+ cells. We are in the process of determining the role of JAK/STAT pathway .in this process. Functional studies will involve blocking of TPO/c-mpl using TPO-R-specific antibodies and determining its impact on human ES-derived hematopoiesis.

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INTESTINAL TISSUE ENGINEERING: A PRELIMINARY STUDY OF LONG TERM IN VITRO PRIMARY CULTURE OF INTESTINAL EPITHELIAL CELLS

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Clinical conditions of the small bowel such as congenital or surgical resection may affect the functional capability of the mucosal lining of the intestinal tract. Despite recent advances in small bowel transplantation, organ shortages remain an issue. Models that mimic the intestinal niche provide opportunities to evaluate stem cell development. Neointestinal engineering from intestinal epithelial organoid units (IEOU) may be a potential alternative therapy to solve the problems of acute organ shortage and rejection in short bowel syndrome (SBS) patients. A novel in vitro enzymatic digestion method to simulate the intestinal stem cell niche was developed in a rat system. Citrulline, an amino acid exclusively synthesized by intestinal epithelial cells, was analyzed in a primary culture for up to 100 days. Three distinct periods of citrulline levels were observed in the culture system. A strong correlation between citrulline and lactate was demonstrated. Addition of rodent bone marrow derived mesenchymal cells increased citrulline synthesis following long term culture. This cellular niche model and the influence of non-hematopoietic cells should have utility in tissue engineering projects.

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A MOLECULAR PROFILE OF ENDOTHELIAL CELL-DERIVED GROWTH FACTORS THAT REGULATE STEM CELL SELF-RENEWAL

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Several molecular pathways that regulate hematopoietic stem cell (HSC) self-renewal in the mouse have recently been elucidated, including Notch, HOXB4, Wnt and bone morphogenetic protein signaling pathways. However, full elucidation of the intrinsic and extrinsic mechanisms that regulate human HSC self renewal has yet to be realized. As a model for identifying extrinsic factors that stimulate human HSC self-renewal, we have studied the capacity for vascular endothelial cells to support human HSC expansion ex vivo. We have found that primary human brain-derived endothelial cells (HUBECs), unlike all other tissue sources of human ECs we have studied, support a 1-2 log amplification of human bone marrow and cord blood HSCs in short term culture, in the absence of contact (Blood 105:576-83, Blood 100:4433-39). These results indicate that HUBECs are an ideal resource to identify novel soluble growth factors that trigger human HSC self-renewal and expansion. In this study, we applied a comprehensive gene expression strategy to identify the secreted gene products differentially expressed by HUBECs as compared to ECs from a wide range of non-brain tissues, including aorta, pulmonary artery, iliac artery, dermal artery and coronary artery. RNA was isolated from 9 different non-brain ECs and 6 different HUBECs, each of which has been shown by our laboratory to promote human HSC expansion in vitro. Samples were hybridized to Operon Human v.3